REMARKS

Applicant courteously acknowledges the Examiner's withdrawal of prior objections to the specification and the claims and of the prior rejection under under 35 U.S.C. § 112, second paragraph. Applicant respectfully requests reconsideration and reexamination of the pending application.

Claims 1-29 are pending in this application.

Claims 1-13 and 21-29 are withdrawn.

The Office has objected to the application because the "oath or declaration" is defective for containing non-initialed and/or non-dated alterations. (Office Action at 2.) Applicant requests that the Office holds this objection in abeyance until there are allowable claims in this application.

(Please note that Applicant has not responded to the heading "Response to Arguments Concerning Claim Rejections -- 35 U.S.C. § 102 (b)," as Applicant is unaware of any pending rejections of the claimed invention under the cited statute.)

Claim Rejections: 35 U.S.C. § 103(a):

The Examiner rejected claims 14-16 under 35 U.S.C. § 103(a) as "obvious over US 2003/0129733 in view of US 6,617,163." (Office Action at 3.) In particular, the Examiner states that Applicant's prior response failed to adequately address the prior rejection under 35 U.S.C. § 103(a), because "one cannot show nonobviousness by attacking references individually where the rejections are based on combinations of references." (Office Action at 2.) In addition, the Examiner summarizes and reasserts the prior rejection as follows:

> Denning et al is one reference used in the rejection. Denning et al teaches a method of mutating cells for locating at least one gene essential for the growth of a haploid fungus, wherein the method of mutating can be

> insertional mutagenesis. The reference that teaches the impala 160 is US 6,617,163, which teaches the use of the *impala 160* transposon for insertional mutagenesis in *Aspergillus fumigatus.* Therefore, in combining the references, impala 160 is taught for mutating cells for locating at least one gene essential for the growth of a haploid fungus. Since the impala 160 transposon was known for use in insertional mutagenesis, it would have been obvious to combine this reference with Denning et al.

(Office Action at 4.) Applicant respectfully traverses.

According to the M.P.E.P., "A rationale to support a conclusion that a claim would have been obvious is that all the claimed elements were known in the prior art and one skilled in the art could have combined the elements as claimed by known methods with no change in their respective functions, and the combination would have yielded nothing more than predictable results to one of ordinary skill in the art." M.P.E.P. § 2143.02. Applicant finds that the Examiner has made such an assertion in this case. This assertion lacks a critical element, however, as prior art can be modified or combined to reject claims as *prima facie* obvious, only as long as there is a reasonable expectation of success. *See id.* Such expectation is lacking in this case, particularly when one considers the prior art as a whole. Therefore, any argument that a case of *prima facie* obviousness of the claimed invention exists must ultimately fail.

The claimed invention, comprises: A method for locating at least one gene essential for the growth of a haploid fungus, said method comprising the following successive steps: (A) providing a fungal haploid strain; (B) generating a diploid strain from the fungal haploid strain; (C) subjecting the diploid strain to *in vivo* transposon mutagenesis in the presence of *impala160* transposon to form one copy of an *impala160* tagged mutation integrated into the genome of the diploid strain; (D) subjecting the mutated diploid strain formed in (C) to conditions for generating a haploid strain, wherein the absence of haploid progeny is indicative of mutagenesis occurring in

a gene essential for growth of the haploid fungus; and (E) locating the gene essential for growth in the fungal haploid strain.

According to the Examiner, Denning *et al.*, teaches a method for locating a gene essential for growth, and U.S. Patent No. 6,617,163 teaches the use of *impala 160* for insertional mutagenesis. Moreover, only when combined do these teachings render the claimed invention obvious. (Office Action at 4.)

In view of the state of the art at the time of invention, however, a person of ordinary skill would not have had a reasonable expectation of success in combining the prior art as the Examiner suggests. For example, although Villalba *et al.*, *Transposon impala, a Novel Tool for Gene Tagging in the Rice Blast Fungus Magnaporthe grisea*, 14(3) Molecular Plant-Microbe Interactions 308, 313 (2001), states that the *impala* 160 transposon "will be very useful for insertional mutagenesis in fungi," it also teaches that *impala* 160 induced mutagenesis does not appear to be random in *M. grisea* and may even depend upon the original insertion site (*id.* at 311-312.). This chararacteristic would likely frustrate attempts to use insertional mutagenesis as a tool for isolating genes essential for any particular pathway, because such searches depend upon random selection processes. *See* Firon *et al.*, *Identification of Essential Genes in the Human Fungal Pathogen Aspergillus fumigatus by Transposon Mutagenesis*, 2(2) Eukaryotic Cell 247, 253 (2003). At the very least, these teachings indicate that the use of *impala* 160 for insertional mutagenesis would have lead to an unpredictable result.

Furthermore, Li Destri Nicosia M. G., *et al.*, *Heterologous transposition in Aspergillus nidulans*, 39(5) Mol Microbiol. 1330, 1339 (2001), also teaches that *impala* induced mutagenesis is non-random and even appears context dependent. The

reference even shows that a tendency exists for the transposon to insert in noncoding regions, thereby leading to a lower mutagenesis rate than expected.

In combination, therefore, these references would teach a person of ordinary skill that one would not have a reasonable expectation of success combining the references as the Examiner suggests. In fact, the results would have been unpredictable. Thus, a *prima facie* case has not been established showing that claims 14-16 are obvious under 35 U.S.C. § 103.

Furthermore, as noted in Applicant's previously filed Amendment, secondary indicia demonstrate that the claimed invention would not have been obvious. According to the M.P.E.P., "evidence rising out of the so-called 'secondary considerations' must always when present be considered en route to a determination of obviousness." *See* M.P.E.P. § 716.01(a) (quoting *Stratoflex, Inc. v. Aeroquip Corp.*, 713 F.2d 1530, 1538 (Fed. Cir. 1983). Secondary considerations may include "evidence of criticality or unexpected results, commercial success, long-felt but unsolved needs, failure of others, skepticism of experts, etc." *See* M.P.E.P. § 716.01(a).

According to the M.P.E.P., "[e]vidence of unexpected properties may be in the form of a direct or indirect comparison of the claimed invention with the closest prior art which is commensurate in scope with the claims." *See* M.P.E.P. § 716.02(b)III (emphasis added). Applicant's prior Amendment contained one form of such evidence, appearing as unexpected results (January 14, 2008, Reply to Office Action at 16-18). In the prior response, Applicant compared the claimed invention with the closest prior art, Denning *et al.* (January 14, 2008, Reply to Office Action at 16-18), and highlighted the differences between them. In particular, Applicant previously noted that Denning *et al.*

teaches the identification of genes essential for growth in fungi, including the use of plasmid insertion mutagenesis. *Id.* The reference does not, however, teach the use of transposons, or even the *impala* 160 transposon. As a result, the presently claimed invention possesses significant advantages over the closest prior art, because "the protocols used prior to Applicant's invention generated a significant number of deletions and genomic rearrangements," and "[t]hese detrimental characteristics would impede the use of such protocols in high throughput assays." *Id.* at 17. The claimed invention, meanwhile, "provides a fast, efficient, and random method for identifying genes essential for growth." *Id.*

"It is improper to combine references where the references teach away from their combination." M.P.E.P. § 2145(D)(2) (citing *In re Grasselli*, 713 F.2d 731, 743, (Fed. Cir. 1983). Moreover, "[t]he totality of the prior art must be considered, and proceeding contrary to accepted wisdom in the art is evidence of nonobviousness." *Id.* (citing *In re Hedges,* 783 F.2d 1038 (Fed. Cir. 1986). In addition to the above cited evidence showing secondary indicia of nonobviousness, the prior art also contains evidence that teaches away from combining the art cited by the Examiner and from using the *impala* 160 transposon in *A. fumigatus*.

According to the state of the art at the time of invention: "[a] random insertional mutagenesis tool should combine: ease in the production of insertional mutants, absence of rearrangements along the genome, and a random distribution of insertions along the genome. *See* Firon *et al.* at 253 (emphasis added). Prior art shows that at least the last trait is lacking for *impala* 160 transposons in filamentous fungi, such as *A. fumigatus*, and, as a result, teaches away from its use in the currently claimed

invention. For example, Villalba *et al.*, teaches that *impala* 160 induced mutagenesis does not appear to be random in *M. grisea* and may even depend upon the original insertion site. Villalba *et al.* at 311-12. Li Destri Nicosia M. G., *et al.*, meanwhile, teaches that *impala* 160 insertions are not entirely random in *A. nidulans* and that the transposon shows a tendency to land outside of open reading frames. Nicosia M. G., *et al.* at 1339. In combination, therefore, these references teach away from use of the the *impala* 160 transposon for insertional mutagenesis. And when added to Applicant's previously cited evidence of unexpected results, these teachings demonstrate that the claimed invention is not obvious.

For the foregoing reasons, as well as those noted previously, claims 14-16 are not obvious under 35 U.S.C. § 103. Accordingly, these grounds for rejection may be withdrawn.

Claim Objections:

The Examiner objected to claims 17 and 19 for depending from a rejected base claim. The claims are amended to include all limitations of the base claim, as suggested by the Examiner. Claim 20 has been similarly amended. Thus, these claims are allowable.

Applicant respectfully requests that this Amendment under 37 C.F.R. § 1.116 be entered by the Examiner, placing claims 14-16 and 18-20 in condition for allowance. Applicant submits that the proposed amendments of claims 18-20 do not raise new issues or necessitate the undertaking of any additional search of the art by the Examiner, since all of the elements and their relationships claimed were either earlier

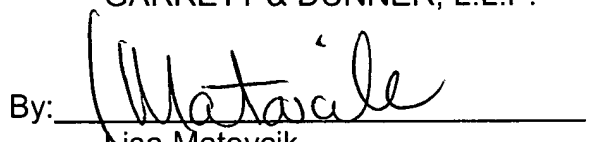
claimed or inherent in the claims as examined. Therefore, this Amendment should allow for immediate action by the Examiner.

Furthermore, Applicant submits that the entry of the amendment would place the application in better form for appeal, should the Examiner dispute the patentability of the pending claims.

Applicant, therefore, requests the entry of this Amendment, the Examiner's reconsideration and reexamination of the application, and the timely allowance of the pending claims.

Please grant any extensions of time required to enter this response and charge any additional required fees to our Deposit Account No. 06-0916.

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

By: 

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Dated: July 17, 2008

Attachments:

Li Destri Nicosia M. G., *et al.*, *Heterologous transposition in Aspergillus nidulans*, 39(5) Mol Microbiol. 1330 (2001).

Molecular Microbiology (2001) 39(5), 1330–1344

Heterologous transposition in *Aspergillus nidulans*

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Summary

***Aspergillus nidulans* is one of the model ascomycete fungi. Transposition events have never been described in this organism. We have determined that this organism has at least 13 copies of a Fot1-related element. These copies are transcribed, non-methylated and polymorphic in various wild isolates. In spite of this, we have failed to isolate transposon insertions when the resident *niaD* gene is used as a transposon trap. This contrasts with the situation described previously in *Fusarium oxysporum*. We show that two elements of *F. oxysporum*, *Fot1* and *impala*, transpose efficiently in *A. nidulans*. We have developed the *impala* system by tagging it with the *yA* gene. This permits the visual detection of the transposon by the colour of the conidiospores. We demonstrate that no endogenous transposase of *A. nidulans* is able to act in *trans* on a defective *impala* element, whereas its own transposase driven by two different promoters is able to mobilize this element. The frequency of excision of these modified elements is between 10^{-4} and 10^{-5}. Loss of the transposable element occurs in about 10% of all excision events. In the remaining 90%, the transposon seems to be integrated at random positions in the genome. The availability of mitochondrially inherited mutations has allowed us to demonstrate that hybrid dysgenesis is apparently absent in *A. nidulans*. The development of this system opens the way to investigating the mechanism underlying the paucity of transposition events leading to visible phenotypes. It should allow us to develop efficient gene-tagging tools, useful in this and other fungi.**

Accepted 14 December, 2000. *For correspondence. E-mail scazzocchio@igmors.u-psud.fr; Tel. (+33) 1 69 15 63 56; Fax (+33) 1 69 15 78 08. †Present address: Dipartimento di Agrochimica ed Agrobiologia, Università di Reggio Calabria, Piazza S. Francesco di Sales 4, 89061 Gallina, Reggio Calabria, Italy. ‡The first two authors have contributed equally to this work.

Introduction

Since the publication of the review by Pontecorvo *et al.* (1953), the fungus *Aspergillus nidulans* has been a paradigm of eukaryotic microbial genetics. A number of groups have isolated spontaneous and induced mutations in hundreds of genes. The number of mutations sequenced by different groups is no doubt in the hundreds. A striking example is the mutational analysis carried out by Arst and colleagues on the *areA* gene, in which the sequence of more than 50 mutations was determined (for example, see Wilson and Arst, 1998). In no case was a sequenced mutation caused by the insertion of a transposon. We have tried to use the *nirA* and *prnA* genes as transposon traps with no success (F. Roussel and C. Scazzocchio, unpublished results). Others report the same lack of success with the *niaD* gene (J. R. Kinghorn, unpublished results). Recently, a number of putative or actually active transposons have been described in a number of fungi, such as *Fusarium oxysporum* (Daboussi *et al.*, 1992; Daboussi and Langin, 1994; Langin *et al.*, 1995), *Magnaporthe grisea* (Farman *et al.*, 1996) and *Tolypocladium inflatum* (Kempken and Kuck, 1996), but also including *Aspergillus niger* (Glayser *et al.*, 1995; Amutan *et al.*, 1996). Close to the *brlA* gene of *A. nidulans*, there is an intact transposon, highly similar to the *Fot1* element of *F. oxysporum* (Kupfer *et al.*, 1997). The sequence of a second such element has been obtained. The putative transposase gene of this element has an open reading frame (ORF) showing 36% identity to the cognate gene of *F. oxysporum*.

The development of transposition in the laboratory strain of *A. nidulans* will provide the methodology for comprehensive gene tagging, an extremely useful tool for genome analysis. This development will also permit the genetic analysis of the transposition process in the model organism rather than in other, less tractable fungi. The recent development of methods to visualize the *in vivo* DNA binding of regulatory proteins and to investigate chromatin structure (Gonzalez and Scazzocchio, 1997; Wolschek *et al.*, 1998; Muro-Pastor *et al.*, 1999) has increased the panoply of tools available in *A. nidulans*. We thus felt encouraged to investigate whether transposition

can occur in this organism or whether it is impossible, presumably because essential host factors could be missing.

The *A. nidulans niaD* gene, coding for nitrate reductase, has been used as a transposon trap in *F. oxysporum* (for a review, see Daboussi and Langin, 1994). We then have a ready-made, simple methodology to study heterologous transposition. We constructed a strain deleted for *niaD*, but not for *niiA*, the adjacent gene coding for nitrite reductase. We then reintroduced the *A. nidulans niaD* gene, which had been inactivated by the insertion of a *Fusarium* transposon *in vivo*. Strains carrying any of these constructions will be unable to grow on nitrate unless the transposon is excised. This phenotypic excision test will be illustrated later in the text for two of our constructions. All *A. nidulans niaD* inactivation events resulting from transposon insertion in *F. oxysporum* carry the original transposon insertions outside the ORF, so that excision events will not result in mutations caused by frameshifts. All excision events can then be detected (Daboussi and Langin, 1994). We have tried this strategy for two transposons, a *Fot1* element (Daboussi *et al.*, 1992) inserted into an intron of the *niaD* gene and the *impala* element (Langin *et al.*, 1995; Hua-Van *et al.*, 1998) inserted into the *niaD* promoter region (Fig. 1). We show that heterologous transposition is possible at high frequency in *A. nidulans*. Both elements have been shown to be autonomous in *F. oxysporum*, and *impala* has been shown to be active in the closely related *Fusarium moniliforme* (Hua-Van *et al.*, 2000). Heterologous transposition across different genera has not been shown before in fungi for DNA transposons, but it has been shown recently for retrotransposons (Nakayashiki *et al.*, 1999). We have also developed a transposition system convenient for both gene inactivation and investigating genomic position effects in this microorganism by constructing a heterologous transposon tagged with an easily scorable autonomously expressed gene.

Results

Attempts to use the A. nidulans niaD *and* nirA *genes in a strain other than the laboratory strains as transposon traps*

We wanted to determine whether the absence of transposition events in known genes is a characteristic of the laboratory strains, all derived from the standard Glasgow strain (Pontecorvo *et al.*, 1953). We selected 63 *niaD* and 17 *nirA* spontaneous mutations by chlorate resistance (Cove, 1976) in the Birmingham wild-type strain G0068 (Pontecorvo *et al.*, 1953). We investigated the presence of insertions by amplifying by polymerase chain reaction (PCR) segments of the appropriate gene in each mutant. Both genes were scanned entirely using appropriate primers (see *Experimental procedures*). No insertion event was detected.

A Fot1*-like element is present in multiple copies in* A. nidulans*, is not methylated and shows position polymorphism*

We constructed by PCR a probe for the transposase of the *Fot1*-like element (Kupfer *et al.*, 1997; see *Experimental procedures*). We probed *Eco*RI-restricted DNA from the Glasgow strain and three additional strains. As there are no *Eco*RI sites in the two sequenced *Fot1*-like transposons, the number of bands should reflect the number of copies present in each strain. Figure 2 shows that the laboratory strain has about 13 copies of the *Fot1*-like element and that the position of these is polymorphic among different strains. DNA methylation is not supposed to occur in *A. nidulans* (Tamame *et al.*, 1983). However, it cannot be excluded that it may occur in specific limited regions of the genome. We investigated the methylation of CG pairs by digesting genomic DNA of a standard laboratory strain. We restricted the genomic DNA with two pairs of isoschizomeric enzymes (*Sau3*–*Nde*II and *Hpa*II–*Msp*I) and probed the resulting Southern blots with an internal sequence of the *Fot1*-like element, amplified on genomic DNA by primers deduced from the transposon sequence (see *Experimental procedures*). We did not find any differences in the cognate digests (not shown), and this establishes that CG sequences in the *Fot1*-like elements of *A. nidulans* are not methylated.

Fot1 *transposes in* A. nidulans

We have introduced by co-transformation with a pPL5 plasmid, carrying the *riboB* gene, the plasmid pEC62, which contains a *niaD* gene interrupted by *Fot1* (1928 bp) in the third intron of the gene (Migheli *et al.*, 1999), in

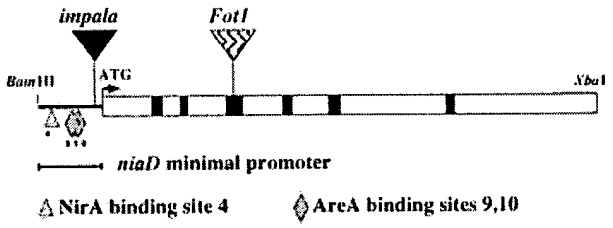

Fig. 1. *A. nidulans niaD* transgene showing the position of the *F. oxysporum* transposable elements. The gene is driven by a minimal promoter sufficient for its expression in both organisms. The extant regulatory sites, NirA4, AreA9 and 10, are shown (Punt *et al.*, 1995). *Fot1* is in the third intron (Daboussi *et al.*, 1992), and *impala* is 10 bp upstream from the initial ATG (Langin *et al.*, 1995). The plasmids that carry these interrupted *niaD* sequences are pEC62 and pNI160 respectively.

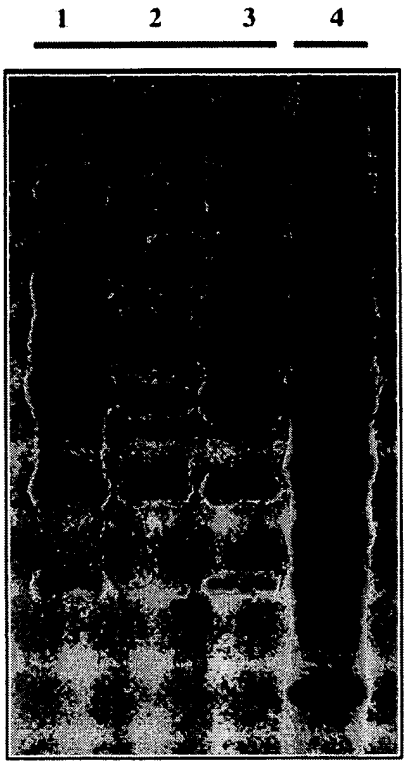

Fig. 2. Polymorphism of the *Fot1*-like elements in different wild-type strains of *A. nidulans*. The DNAs were restricted with *Eco*RI and probed with the PCR-amplified ORF corresponding to transposase of the *Fot1*-like element of *A. nidulans* (Kupfer *et al.*, 1997; see *Experimental procedures*). 1, Birmingham wild-type strain *G001*; 2, Birmingham wild-type strain *G0068*; 3, Birmingham wild-type strain *AJC10.16*; 4, Glasgow laboratory strain.

A. nidulans strain CS2708 (*yA2*, *riboB2*, *pantoB100*, *niaDΔ353*), in which *niaDΔ353* is a deletion of 2786 bp from bp −55 to bp +2728 (in relation to the ATG). This deletion overlaps the site of insertion of the transposon and precludes the appearance of *niaD*$^+$ strains by recombination between the resident *niaD* sequences and sequences of the transgene. The recipient strain carried a *riboB2* mutation, and plasmid pPL5, carrying a wild-type *riboB* gene, was used to select co-transformants. We isolated six transformants and, among those, three were able to revert to the ability to use nitrate (phenotypic excision test; Migheli *et al.*, 1999; see *Experimental procedures* and Table 1). Southern blots (not shown) carried out on the original transformants and 18 revertant strains and probed both with *Fot1* and *niaD* sequences showed that all transformants carried copies of the *Fot1*-inactivated *niaD* transgene and that a non-interrupted *niaD* gene is present in all nitrate-using revertants. In all three original transformants, the inserted plasmid was present in multiple copies. We obtained by outcrossing a strain carrying only one copy of the *niaD* gene interrupted by *Fot1*, which reverts to *niaD*$^+$ at a

Table 1. Transgene copy number and transposition frequencies of strains carrying the *niaD* transgene interrupted by the *Fot1* transposable element.

Strain	Transgene copy number	Excision frequency
Trf1[a]	≈ 10	1.10^{-6}
Trf2	≈ 20	5.10^{-7}
Trf3	Not determined	1.10^{-6}
CS2767[b]	1	1.10^{-7}

a. Trf indicates primary transformants carrying an *niaD* gene interrupted by *Fot1*.
b. CS2767 was obtained by outcrossing Trf2.
Copy number and excision frequencies were determined as indicated in *Experimental procedures*.

frequency of 10^{-7} (CS2767 strain; for details, see *Experimental procedures* and Table 1).

Figure 3 shows Southern blots of the CS2767 *niaD*$^-$ strain carrying a single-copy interrupted transgene and seven revertants selected at random. Probing with a *niaD*

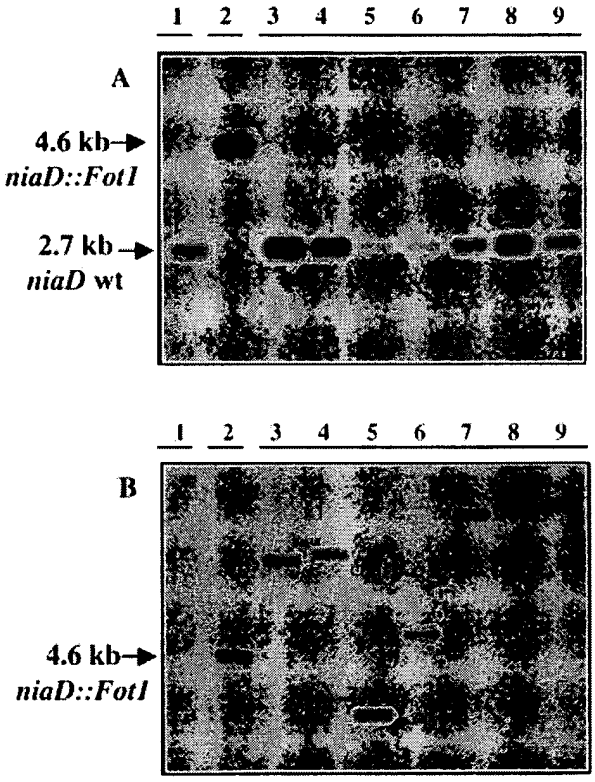

Fig. 3. Southern blots of genomic DNA isolated from the CS2767 strain harbouring one copy of the *niaD* transgene interrupted by *Fot1* and its *niaD*$^+$ revertants. 1, *A. nidulans* wild-type strain; 2, strain carrying one copy of the *niaD* transgene interrupted by *Fot1*; 3–9, *niaD*$^+$ revertants. DNAs restricted with *Eco*RI.
A. Hybridization with the *niaD* probe: the arrow indicates the 2.7 kb *Eco*RI fragment of the *niaD* gene, corresponding to the functional wild-type gene, and the gene reconstituted after transposon excision.
B. Hybridization with the *Fot1* probe: the arrow indicates the 4.6 kb *Eco*RI fragment of the *niaD* gene interrupted by *Fot1* in the transformant strain. 8, this revertant has lost the *Fot1* transposable element.

probe demonstrates the reconstitution of the cognate gene. Probing with a *Fot1* sequence demonstrates that the *Fot1* element is reinserted in the genome, in each case at a different position. One excision event (see Fig. 3, column 8) results in the loss of the transposon.

Both the heterologous Fot1 *element and the resident* Fot1*-like elements yield transcripts of the correct length*

Figure 4A shows a Northern blot comparing the transcription of *Fot1* in *F. oxysporum* and *A. nidulans*. It can be observed that transcription of *Fot1* is much more efficient in *A. nidulans* than in *F. oxysporum* itself. The transcript is not even detectable in a strain of *F. oxysporum* carrying two copies of *Fot1* (Deschamps *et al.*, 1999). This suggests that fungi may have evolved systems of specific repression for their resident transposons. We have therefore reprobed the same membrane with a probe that should reveal the transcript of the resident *A. nidulans* *Fot1*-like elements. We clearly detected a transcript of the predicted length (1.6 kb) for the putative transposase gene in the laboratory strain of *A. nidulans*. This shows that the introduction of as many as 20 copies of the heterologous transposon does not repress the transcription of the resident *Fot1*-like copies (not shown). This is confirmed by an independent experiment (Fig. 4B).

The Mariner*-like element* impala *transposes in* A. nidulans

To detect whether the *impala* element is able to transpose in *A. nidulans*, we followed an identical strategy to that detailed above for *Fot1*. A 1280 bp *imp160* element of the E subfamily of *F. oxysporum* has jumped in the promoter of the *niaD* gene (Hua-Van *et al.*, 1998). This element was shown previously to be autonomous in *F. oxysporum* (Hua-Van *et al.*, 2000). The *niaD* gene carrying this transposon was introduced in a strain of *A. nidulans* deleted for *niaD* as described above. We isolated 11 co-transformants able to revert to *niaD*$^+$ (see *Experimental procedures*). Reversion occurs at frequencies ranging from 10^{-2} to 10^{-7} (Table 2).

We attempted to isolate by outcrossing a strain carrying only one copy of the transgene interrupted by *impala*. We were unable to do this, but a strain carrying one intact copy of the transgene and a second showing internal rearrangements, including deletions of the transposase gene, was isolated. This second copy is presumably inactive. We have thus used this strain to investigate the mobility of the *impala* element. In this strain, *impala* transposes at a frequency of 10^{-7} (strain CS2780; Table 2). Figure 5A shows that each excision event is accompanied by reinsertion of the transposon at a different position in the genome. No loss of the transposon was detected in the revertants tested. The same Southern blot was reprobed with a *niaD* probe and, in every case, we detected the reconstitution of a wild-type *niaD* gene, as shown before for *Fot1* (not shown).

Footprints left by transposon excision

We have sequenced six excision events in a strain in which *Fot1* has excised and, in all cases, we found that excision left a ATTA footprint. For *impala*, we sequenced five different excision events. Three correspond to CTGTA, one to CATA and one to CAGTA excision events. The TA sequence corresponds to a duplication of the target sequence.

Construction and transposition of a tagged impala *transposon*

The *yA* gene of *A. nidulans* encodes a laccase catalysing the conversion of yellow to green pigment. Moreover, the gene is expressed in phialides during conidiation, and its expression is autonomous in different conidiophores (O'Hara and Timberlake, 1989). All uninucleate conidia in one phialide descend from the same nucleus. This autonomy of expression of genes involved in conidial pigmentation has been the basis for much of the parasexual genetics of *Aspergillus* and provides a simple visual method for detecting the presence and eventual loss of the transposable element. We therefore inserted the 3543 bp *yA* gene with (presumably) the complete promoter (Aramayo and Timberlake, 1990) downstream from the ORF of the *impala* element (*imp160*; Hua-Van *et al.*, 1998) present, as above, in the promoter of the *niaD* gene. This construction, named *impala:: yA*, is 4823 bp long as opposed to the 1280 bp of the original transposon. The plasmid carrying this construction was named pCM3 (Fig. 6; see *Experimental procedures*).

We used the pMC3 plasmid to co-transform two different strains: CS2708 *yA2*, *riboB2*, *pantoB100*, *niaDΔ353 and* CS2752 *yAΔ::pyr4*, *niaDΔ353*, *riboB2*, *argB2*, *pabaA1*. The first strain carries a standard *yA*$^-$ mutation. The second strain carries a newly constructed (see *Experimental procedures*) deletion of the whole *yA* sequence and, thus, any recombination between host and plasmid sequence is precluded.

From the first strain CS2708, we isolated nine *yA*$^+$ (green conidiating) co-transformants by transformation with pCM3 and pPL5, the latter carrying a *riboB* gene. Thus, the *yA* gene inserted in *impala* is correctly expressed. Furthermore, all these transformants reverted to a *niaD*$^+$ phenotype at frequencies between 10^{-4} and 10^{-5} (phenotypic excision test; see *Experimental procedures*). Southern blots showed that two of these, Triy100 and Triy101, carry only one copy of pCM3 (not shown). These strains revert at a frequency of about 10^{-5}

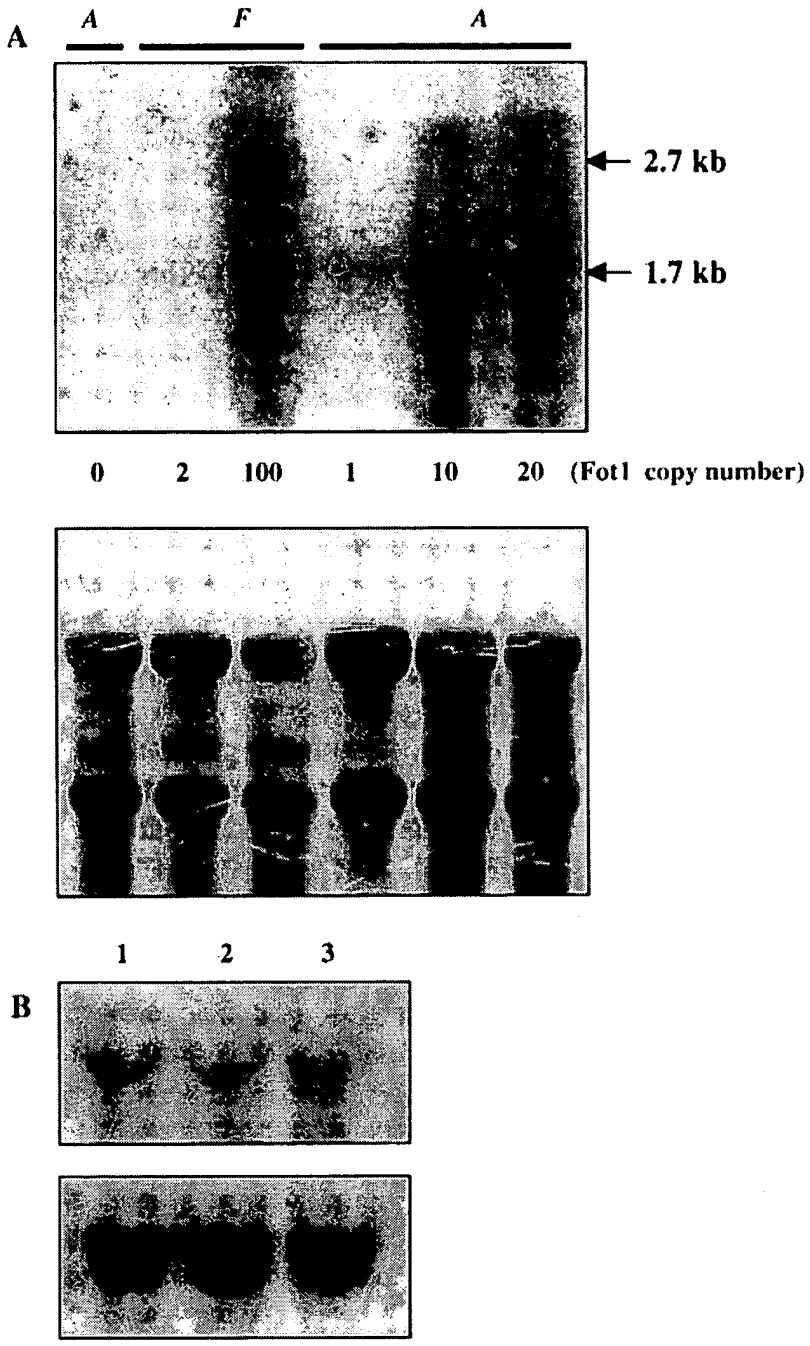

Fig. 4. Northern hybridization of total RNAs extracted from *A. nidulans* and *F. oxysporum* strains.
A. The mRNA corresponding to the *Fot1* ORF. Hybridization with the *Fot1* probe. Letters above the blot: A, *A. nidulans;* F, *F. oxysporum.* Numbers below the blot indicate the *Fot1* copy number for each strain analysed, thus indicating the *A. nidulans* recipient strain carrying no exogenous copies of *Fot1*. The different wild-type strains of *F. oxysporum* (*Fot15* and *niaD-37*; Deschamps *et al.*, 1999) carry two and 100 copies respectively. The *A. nidulans* strains are those shown previously in Table 1. The arrows indicate the 1.7 kb *Fot1*-specific transcript and the 2.7 kb transcript resulting from the *niaD37* insertion event, which corresponds to a chimeric *niaD–Fot1* transcript (Deschamps *et al.*, 1999). The lower blot is coloured with methylene blue.
B. The mRNA corresponding to the ORF of the *A. nidulans Fot1*-like element. Top, hybridization with the probe corresponding to the relevant ORF (see *Experimental procedures*). Bottom, the same membrane hybridized with an *acnA* (actin) probe (Fidel *et al.*, 1988). 1, recipient strain, carrying no exogenous *Fot1* copies; 2, transformant harbouring 10 copies of *F. oxysporum Fot1* (Tr1); 3, transformant harbouring 20 copies of *F. oxysporum Fot1* (Tr2) (see Table 1).

(Table 3). Of these revertants, about 10% were yA^-, indicating loss of the transposable element (see below). We demonstrated by Southern blot analysis that the $niaD^+$ phenotype was the result of the *impala::yA* element excision. Southern blots of six yA^+ revertants obtained from Triy100 show that, in all six yA^+ revertants, the transposon has integrated at different locations of the genome and that an intact *niaD* transgene has been restored by the excision of the transposon (Fig. 5B and C). From strain CS2752, 77 green co-transformants were isolated. Transformants carrying only one copy of the integrated plasmid could be identified in principle because they should yield at least some yA^- revertants, those in which the unique copy of the transposable element is lost. Two transformants showed this characteristic: Triy58 and Triy100. The transposition frequency and the copy number were determined for a number of the strains described above. The results (Table 3) show that transposon copy number is not obviously correlated with excision frequency. This implies that transposition may

Table 2. Transgene copy number and excision frequencies of strains carrying the *niaD* transgene interrupted by the *impala* transposable element.

Transformant	Transgene copy number	Excision frequency
Tri1[a]	3	1×10^{-2}
Tri2[a]	2	5×10^{-3}
Tri3[a]	2	1×10^{-3}
Tri4[a]	≈15	1×10^{-3}
Tri5[a]	≈15	1×10^{-3}
Tri6[a]	≈15	2×10^{-3}
Tri7[a]	≈ 2	1×10^{-4}
Tri8[a]	6	5×10^{-4}
Tri9[a]	4	1×10^{-4}
Tri10[a]	2	1×10^{-4}
Tri11[a]	3 (in tandem)	4×10^{-4}
CS2780[b]	1 + 1[c]	1×10^{-7}

a. Tri, primary transformants.
b. Obtained by outcrossing Tr11.
c. One copy + one additional copy with internal rearrangements.

depend on the site of insertion. In one transformant shown in Table 3, Triy2, no excision was detected. We have shown by Southern blots (not shown) for this and all other transformants listed in Table 3 that integration has occurred through heterologous recombination of the sequences of the plasmid and that the transgenes are intact.

We have verified that the phenotype of the *yA*$^-$ revertants obtained from strain CS2773 (defined in Table 3) resulted from loss of the tagged transposon. To this end, pools of up to 26 *yA*$^-$ revertants were grown, the DNA extracted and the presence of *impala* checked by amplification with suitable primers (see *Experimental procedures*). Positive results in reconstruction experiments, in which a strain carrying one copy of *impala* was included in the pool, showed that the method is adequate.

Table 3. Transgene copy number and excision frequencies of strains carrying the *niaD* transgene interrupted by the *impala::yA* transposable element.

Strains	Transgene copy number	Excision frequency
Triy2[a]	2 (in tandem)	$< 10^{-7}$[b]
Triy3[a]	2 (in tandem)	2×10^{-5}
Triy8[a]	2 (in tandem)	3×10^{-5}
Triy13[a]	3	1×10^{-3}
Triy27[a]	4	6×10^{-6}
Triy29[a]	2	4×10^{-5}
Triy40[a]	10 (in tandem)	7×10^{-4}
Triy56[a]	10 (in tandem)	2×10^{-3}
Triy58[a]	1	4×10^{-6}
Triy100[c]	1	5×10^{-5}
Triy101[c]	1	4×10^{-5}
CS2773[d]	1	6×10^{-5}
CS2778[e]	1	4×10^{-5}

a. Triy 2–58 were obtained in strain CS2752.
b. No *niaD*$^+$ revertants obtained.
c. Triy100 and 102 were obtained in strain CS2708 (see *Experimental procedures*).
d. CS2773 strain was obtained by outcrossing Triy100 to a *yA* deleted strain (CS2753).
e. *impala::yA* inserted in the resident promoter of *niaD* (*niiA–niaD* gene cluster; see text).
This strain, selected for further work, is one of four identical strains obtained in the transformation procedure (see text and *Experimental procedures*).

A total of 367 *yA*$^-$ revertants were analysed as above, and in no case did we observe amplification of *impala* sequences (not shown).

Impala *is excised at high frequency when integrated in the* niiA–niaD *intergenic region in chromosome VIII*

The results in the previous section suggest very strongly

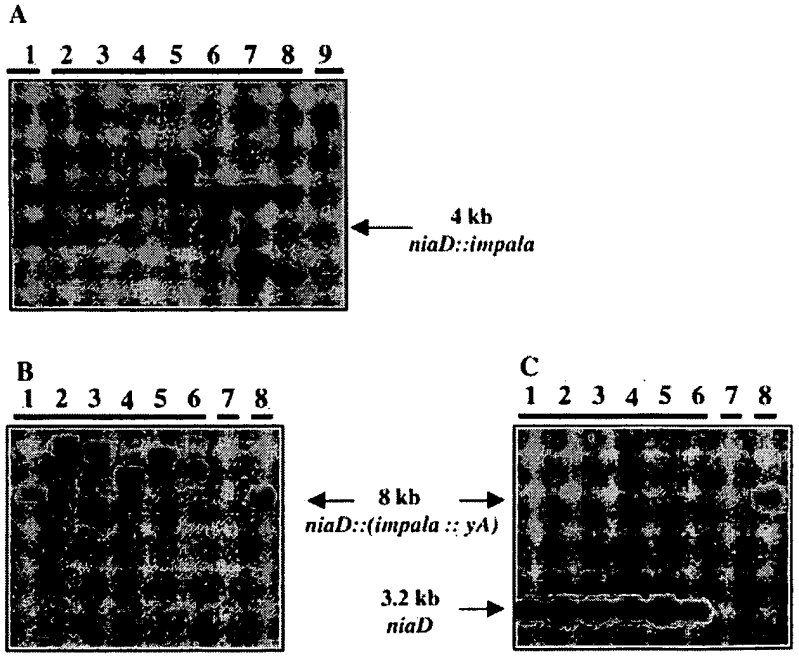

Fig. 5. A. Southern blot of *Eco*RI-digested genomic DNA isolated from the CS2780 transformant harbouring one copy of the *niaD* transgene interrupted by *impala* and one additional copy with internal rearrangement (shown in Table 2) and its *niaD*$^+$ revertants. The blot was probed with the ORF of *impala* (see *Experimental procedures*). 1, CS2780 transformant harbouring one intact copy of the *niaD* transgene interrupted by *impala*; the arrow indicates the 4 kb *Eco*RI fragment of the *niaD* gene interrupted by *impala*; 2–8, *niaD*$^+$ revertants; 9, *A. nidulans* recipient strain.
B. Southern blot of a *Sph*I-digested genomic DNA isolated from the Triy100 transformant harbouring one copy of the *niaD* transgene interrupted by *impala::yA* (see Table 3) and its *niaD*$^+$ revertants. The blot was probed with *impala* (see *Experimental procedures*). 1–6, *niaD*$^+$ revertants; 7, CS2708 recipient strain; 8, Triy100 *impala::yA* original transformant; the arrow indicates the 8 kb *Sph*I fragment of the *niaD* gene interrupted by *impala::yA*.
C. As (B) but probed with *niaD* sequence (see *Experimental procedures*).

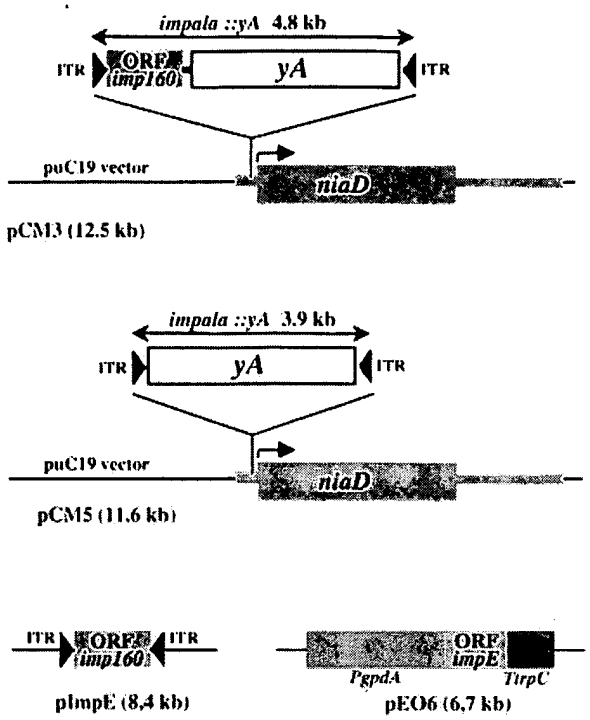

Fig. 6. Constructions containing autonomous or defective *yA*$^+$ tagged *impala* elements. Top: *A. nidulans niaD* transgene interrupted by the *F. oxysporum impala* transposable element (*imp160*; Langin *et al.*, 1995) tagged with the *A. nidulans yA* gene driven by its own promoter (Aramayo and Timberlake, 1990). The tagged element is in the position in the *niaD* promoter previously shown in Fig. 1. This autonomous transposable element was constructed by cloning the *yA* gene downstream of the *impala* ORF and upstream of its righthand inverted terminal repeat (ITR). This construction in puC19 yields the pCM3 plasmid. Bottom: in the defective transposable element, the *yA* gene substitutes for most of the *imp160* ORF (resulting plasmid pCM5). Below, we show the two constructions placed *in trans and* used to mobilize the defective element. Left: the whole autonomous *impE* (pImpE plasmid); right: the *impE* transposase placed under the control of the *gpdA* promoter. *P*, promoter; *T*, terminator.

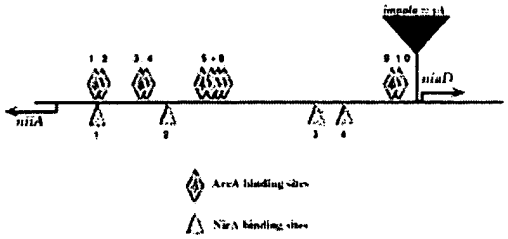

Fig. 7. The *impala::yA* autonomous transposable element integrated in the resident *A. nidulans niiA–niaD* bidirectional promoter (Punt *et al.*, 1995). All the AreA and NirA binding sites are indicated. Compare with Fig. 1.

that the frequency of excision depends upon the site of integration. Thus, we do not know what would be the behaviour of *impala* integrated in the resident *niiA–niaD* gene cluster in chromosome VIII. We therefore inserted by a double recombination event an *impala* transposon carrying the *yA* gene (plasmid pCM3 linearized using *Sph*I) in the *niiA–niaD* intergenic region of the CS2755 *niiA*$^+$ *niaD*$^+$ strain deleted for the *yA* gene (see *Experimental procedures*). This is shown in Fig. 7. We obtained four independent strains that carry the labelled *impala* element exactly in the same position in the *niaD* promoter as in the strains described previously, but within the resident gene cluster rather than in a transgene. As expected, these strains are *yA*$^+$, *niiA*$^+$ *niaD*$^-$, that is they show green conidiospores and are able to grow on nitrite but not on nitrate as sole nitrogen source. The phenotypic excision test showed (strain CS2778) that *impala* is excised at a frequency of 4×10^{-5}, which compares with the best excision frequencies obtained with one of the strains carrying one copy of the *niaD* transgene interrupted by *impala* (strain CS2773; for details, see Table 3). Of the *niaD*$^+$ strains, 84% are *yA*$^+$, again showing a high frequency of reinsertion of the modified transposable element.

The *niaD* transgene used originally as a transposon trap in *F. oxysporum* carried an incomplete promoter, notably lacking the important regulatory upstream activating sequences (UAS) NirA2 and NirA3 (Punt *et al.*, 1995) and the cluster of GATA sites positioned in the nucleosome-free region of the promoter (Muro-Pastor *et al.*, 1999). Not surprisingly, the growth on nitrate obtained after excision of either *Fot1* or *impala* from such a transgene is clearly weaker than that of the wild type. In the construction described here, the whole *niiA–niaD* intergenic region is intact after excision of the transposable element and, as expected, the growth of strains after excision is identical to that of the wild type (Fig. 8).

A. nidulans *does not have a transposase able to complement a defective* impala *element*

We wanted to investigate whether a transposase is able to mobilize in *A. nidulans* a defective *impala*-like transposon. We therefore constructed the plasmid pCM5, similar to pCM3, but in which the *yA* gene substitutes most of the *imp160* ORF (see *Experimental procedures*). The resulting plasmid (pCM5) is shown in Fig. 6. The CS2752 strain was co-transformed with the pCM5 and pFB39 plasmids (the latter carrying the *argB* gene). Four *argB*$^+$ transformants with a *yA*$^+$ phenotype were isolated. We have shown by Southern blots for these transformants that the transgenes are intact (not shown). Approximately 5×10^8 conidia from each transformant were spread on minimal medium with nitrate as nitrogen source. We found that none of them is able to revert to *niaD*$^+$. Thus, there is no endogenous transposase able to activate this defective element.

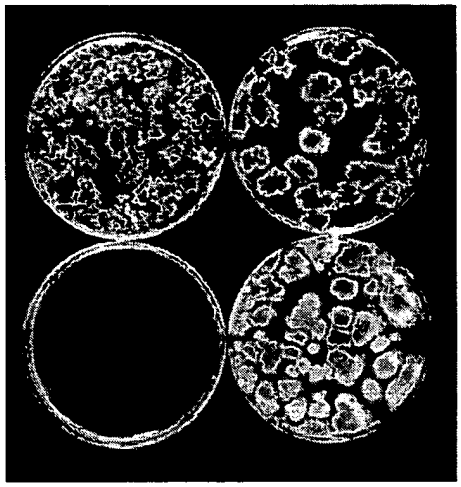

Fig. 8. Phenotypic excision test. All plates contain sodium nitrate as sole nitrogen source. Top right: strain CS2755, *niaD*$^+$, *yAΔ::pyr4*. This strain has yellow conidia and is able to use nitrate as sole nitrogen source. Bottom right: strain CS2753, *niaDΔ353*, *yAΔ::pyr4*. This strain is unable to use nitrate as a nitrogen source. About 10^7 spores were plated. Top left: strain CS2778. This strain carries an *impala* element, tagged with *yA*$^+$ in a *yAΔ::pyr4* background. This element is located in the resident *niaD* promoter (see text). About 10^6 spores were plated. The colonies that are visible are those in which the transposon has jumped out of the *niaD* promoter. Note the green colonies (dark) and some yellow (light) colonies. In the latter, the transposon was lost. Bottom left: as above, but strain CS2773. This strain carries a *niaD* complete deletion and a single copy of an *niaD* transgene, with an incomplete promoter, interrupted by the *impala* element tagged with the *yA*$^+$ gene. About 10^6 spores were plated. Only the colonies in which the transposon has jumped out are able to grow on nitrate. Note the reduced ability of these colonies to use nitrate as a nitrogen source. Also here, some lighter colonies (yellow) are visible, indicating the loss of the *impala* tagged element. For the complete genotypes and history of strains, see text and *Experimental procedures*.

The impala*-defective transposon can be* trans*-activated by its own transposase*

We developed two double-component transposition systems in which the defective tagged copy described above is mobilized by a *trans*-activating element supplying the transposase. In the first system, the *trans*-acting transposase was placed under the control of the constitutive strong *gpdA* promoter (glyceraldehyde-3-phosphate dehydrogenase gene) of *A. nidulans* (see *Experimental procedures*). In the second system, the *trans*-activating element is simply the whole autonomous *impE* transposon placed *in trans* (Hua-Van *et al.*, 1998; 2000).

To assess the efficiency of transposition with the double-component system, the strains were obtained in two stages. First, the CS2752 strain was co-transformed with each *trans*-activating element and with the pFB39 plasmid carrying the *argB*$^+$ gene as described previously. A strain with one copy of the *trans*-activating element was chosen after Southern blot analysis of a number of transformants (not shown). The strain carrying the *impE trans*-activator plasmid in a single copy was called CS2776; the other, carrying the *gpdA*-driven transposase in single copy, was designated CS2777.

The CS2776 and CS2777 strains were then co-transformed by the defective tagged element (plasmid pCM5) and the pPL5 plasmid carrying the *riboB*$^+$ gene as described above. We obtained three and five transformants from strains CS2776 and CS2777 respectively. These strains carry, as the parent strains, a single copy of the *trans*-acting transposase but a variable number of the *niaD* transgene interrupted by the defective *impala* tagged element (see Table 4). Table 4 shows that the defective transposable element carried by the pCM5 plasmid can be *trans*-activated. Excision frequencies are variable, but the higher frequencies observed in strains carrying single copies of the *niaD* interrupted transgene are similar to those shown in Table 3 for the intact tagged *impala* transposon. However, no excision events were obtained in one of the transformants (Triyd1). Southern blots (not shown) of this and all other transformants in Table 4 show that the transgene and transposon elements are intact. This result is in line with the suggestion that excision frequencies depend on the site of insertion. An alternative, albeit very unlikely possibility, is that a mutation, undetectable in Southern blots, has occurred in the *niaD* sequences during the transformation procedure.

No evidence for hybrid dysgenesis in can be found in sexual crosses of A. nidulans

Hybrid dysgenesis is a phenomenon described for some *Drosophila* transposons, in which transposition after fertilization depends on whether the transposable element is carried by the sperm or the unfertilized egg. When the transposable element is carried by the egg, transposition is prevented by a cytoplasmic repressor. When the transposon is carried by the sperm, transposition occurs

Table 4. Excision frequencies obtained with the double-component transposition systems.

	Transformant	Defective transgene copy number	Excision frequency
Transposase driven by the *gpdA* promoter	Triyd1	1	$< 10^{-8}$[a]
	Triyd2	5 (in tandem)	7×10^{-4}
	Triyd3	1	5×10^{-4}
	Triyd4	2 (in tandem)	4×10^{-4}
	Triyd5	1	5×10^{-6}
Transposase driven by its own promoter	Triyd10	2 (in tandem)	7×10^{-4}
	Triyd11	1	1×10^{-4}
	Triyd12	Tandem[b]	6×10^{-3}

a. No *niaD*$^+$ revertants obtained.
b. More than five copies.

Table 5. Investigation of hybrid dysgenesis.

Strains carrying *impala*	Mitotic excision frequency	Cleistothecia in which *impala* is in the male nucleus				Cleistothecia in which *impala* is in the female nucleus			
		No. of cleistothecia	No. of ascospores	No. of excision events	Excision frequency	No. of cleistothecia	No. of ascospores	No. of excision events	Excision frequency
Tri11 Three copies of *niaD* interrupted by *impala*	4×10^{-4}	51	8.4×10^{5}	8	10^{-5}	46	10^{7}	30	2.7×10^{-6}
Triy100 One copy of *niaD* interrupted by *impala::yA*	5×10^{-5}	46	2.0×10^{6}	4	2×10^{-6}	51	10^{6}	3	2.8×10^{-6}
Triy100 One copy of *niaD* interrupted by *impala::yA*	5×10^{-5}	17	2.0×10^{6}	8	3×10^{-6}	14	1.6×10^{6}	7	4.3×10^{-6}

at high frequency (Bregliano *et al.*, 1980). This phenomenon is analogous to the zygotic induction of phage λ (Jacob and Wollman, 1956).

A. nidulans is a homothallic organism but, at the origin of each cleistothecium, each of the two nuclei can behave as either the male or the female gamete. This is shown by the behaviour of both mitochondrial and maternal-effect nuclear genes (Apirion, 1963; Roberts and Orias, 1973; Rowlands and Turner, 1973; Gunatilleke *et al.*, 1975). In particular, when two strains carrying two different alleles of a mitochondrial gene are crossed, all ascospores in all asci in a given cleistothecium carry exclusively, without exception, one and the same allele. This identifies the female parent in the original fertilization event. This phenomenon allowed us to investigate whether hybrid dysgenesis can be obtained with *impala*. This could be of some importance if *impala* is to be used for gene tagging. We therefore crossed strains carrying a deletion of the resident *niaD* gene and one copy of the *niaD* gene interrupted with *impala* to a strain also deleted for *niaD* but carrying the mitochondrial mutation *camA112* as well, resulting in resistance to chloramphenicol (Gunatilleke *et al.*, 1975). The hybrid cleistothecia in which the transposon-carrying parent behaves as the male nucleus will yield only chloramphenicol-resistant progeny, whereas cleistothecia in which the transposon-carrying parent behaves as the female nucleus will yield only chloramphenicol-sensitive progeny. Every single transposition event will be detected by the presence of $niaD^{+}$ ascospores on nitrate as the sole nitrogen source. Three different experiments were carried out. In the first experiment, a transformant carrying multiple copies of the *niaD* transgene interrupted by *impala* was used (Tri11; see Table 2). In the second and third experiments, a strain carrying a single copy of the *niaD* transgene interrupted by *impala::yA* was used (Triy100; see Table 3). In the first and second experiments, all cleistothecia from the two types were pooled and analysed in bulk. In the third experiment, each cleistothecium was analysed individually. In the third experiment for both *impala* 'male' and 'female' fertilization events, the number of excision events per cleistothecium followed that expected from a Poisson distribution with classes in which excision events were either zero, one or two per cleistothecium, and the number of viable ascospores varied between 3×10^{4} and 2×10^{5}. In Table 5, we show the results of the bulk selection of the first and second experiments and the sum for all individual cleistothecia for the third experiment. With the exception of a probably non-significant increase in excision when *impala* is carried in multiple copies in the male nucleus, these experiments show no evidence for hybrid dysgenesis in strains carrying *impala*. Control crosses, in which the *impala* element was absent, showed that the *camA112* allele has no effect on ascospore

viability (not shown). The only visible effect is a lower frequency of excision in the products of sexual crosses compared with the frequency of excision in asexual spores.

Discussion

The results presented above establish that heterologous transposition is possible in *A. nidulans*.

We have demonstrated this for two transposons belonging to different families. *Impala* belongs to the *Tc1-mariner* family (Langin *et al.*, 1995), and *Fot1* belongs to a different family, found in fungi, but related to the human *tigger* and *Drosophila pogo* elements (Daboussi, 1997). With the *impala* transposon, the frequencies of excision are relatively high. The fact that the use of a strong promoter fails to increase transposon excision may indicate that the transcription of the transposase ORF is not the rate-limiting step in this process. However, this is only one possibility; for example, position effects may change excision frequency, and evidence for their operation has been presented here.

Most of the excised transposons reinsert into the genome at locations that are random within the limits of the resolution of Southern blots. Work to be presented elsewhere (C. Brocard-Masson, A. Apostolaki, C. Drevet and C. Scazzocchio, unpublished results) shows, not surprisingly, that *impala* can jump from one chromosome to a second one. The yA^+ tagged *impala* transposon permits us to obtain a quantitative estimate of transposon loss or eventually to detect the insertion of the transposon in regions of the genome where the expression of yA^+ would be silenced. All events analysed up to now indicate loss of the transposon in yA^- revertants. The frequency of transposon loss (between 10% and 20% in different strains) indicates that, as proposed for other DNA non-replicative transposons, transposition occurs through a cut-and-paste mechanism that involves free intermediates. These have been detected for Ac/Ds (Gorbunova and Levy, 2000) and the fungal *Restless* transposon (Kempken and Kuck, 1998).

A number of *Fot1*-like copies are present in the laboratory and other strains of *A. nidulans*. Their polymorphism suggests that these elements are indeed mobile. One could ask why, among the mutations accumulated in more than 50 years of conventional *Aspergillus* genetics, no mutation resulting from a transposon insertion was ever observed. Using the heterologous transposons, $>10^4$ events were observed, and no morphological and only two spore colour mutations were ever obtained. Of these, one was shown by conventional genetic crosses to result from the transposition event, and it maps in the *yB* gene in chromosome VIII (C. Brocard-Masson, A. Apostolaki, C. Drevet and C. Scazzocchio, unpublished results). The paucity of visible mutations resulting from transposon jumping contrasts with the huge number of colonies carrying such mutations that appear after any conventional chemical or ultraviolet (UV) mutagenesis. For example, in a recent UV mutagenesis experiment carried out in the laboratory, 6% of the surviving colonies carried visible colour or morphological mutations. The gene density in *A. nidulans* is quite high. It has been estimated at one gene every 3.2 kb (Kupfer *et al.*, 1997). In a forthcoming article, we shall demonstrate that insertion in known genes is actually possible with the *impala* tagged transposon. This necessitates appropriate selective techniques and also shows that the frequency of transposon insertion is about two orders of magnitude below the spontaneous mutation rate for a given gene (C. Brocard-Masson, A. Apostolaki, C. Drevet and C. Scazzocchio, unpublished results). All these results indicate that, in this species and perhaps in other fungi, transposons have a propensity to land outside ORFs or essential regulatory regions. Indeed, of eight independent insertion events of *impala* and *Fot1* obtained when the *A. nidulans niaD* gene is used as a transposon trap in *F. oxysporum*, none occurred in the ORF but in introns or 5′ regions (Deschamps *et al.*, 1999). However, when the resident *F. oxysporum niaD* gene was used as a transposon trap, all three *Fot1* events obtained were in the ORF (Daboussi and Langin, 1994). R. Prade has pointed out to us that the 'immunity' of genes to foreign DNA insertion may not be limited to transposons. In fact, very few heterologous integration events caused by the insertion of plasmids in transformation experiments result in visible mutations (P. Ayoubi and R. Prade, unpublished results). Examples in which it has happened can be found in Diallinas and Scazzocchio (1989) and Tilburn *et al.* (1990). It will be interesting to establish whether the same mechanism underlies both phenomena.

We have failed to find any evidence for hybrid dysgenesis. This experiment was attempted because the characteristics of the sexual cycle of *A. nidulans* and the availability of clear-cut mitochondrial point mutations makes *A. nidulans* an ideal organism for this type of work. We have been very cautious in the interpretation of this negative result, because there is no evidence as to whether, and if so when, the transposase of *impala*, which derives from an imperfect fungus, is expressed during the sexual cycle. In particular, there is no evidence whether this promoter would be expressed in the zygote. A clear-cut result would have meant that the transposase is expressed in the zygote. This would have been an interesting result in its own right and would have suggested that a cytoplasmic repressor or inhibitor of transposition is present. A negative or ambiguous result calls for further investigation on the developmental regulation of the transposase promoters. The use of a heterologous tagged

transposon in a model organism will permit investigation of the molecular basis of the discrimination shown by transposons in their choice of landing sites, the role of the sequence and chromatin environment in the frequency of excision, the regulation of transposition during the sexual and asexual cycles and eventually the construction of suitable efficient gene-tagging tools for filamentous fungi. Recently, following our results, transposition of modified *impala* transposons has been shown to be active in *Aspergillus fumigatus* (A. Firon, M.-C. Grosjean-Cournoyer and C. d'Enfert, unpublished data) and in *Magnaporthe grisea* (Villalba *et al.*, 2000).

Experimental procedures

Plasmids pFB39 and pPL5 carry the *A. nidulans* genes *argB* (coding for ornithine carbomyltransferase; Buxton *et al.*, 1989) and *riboB* (Oakley *et al.*, 1987) respectively. pFB6 contains a functional *pyr4* gene (coding for orotidine-5′-phosphate decarboxylase) of *Neurospora crassa*. This gene complements the *pyrG89* mutation of *A. nidulans*. pEB06 contains a 6 kb *Nru*I–*Nru*I DNA fragment, which carries a functional *yA* gene flanked by its upstream and downstream regions (Aramayo and Timberlake, 1990).

bAN353 was constructed in order to obtain a complete *niaD* deletion by homologous recombination (see below). It carries a 1.8 kb sequence in which a fragment (0.3 kb *Bam*HI–*Eco*RI) of the *niaD* promoter was ligated to a sequence (1.5 kb *Eco*RI–*Hin*dIII) 3′ to the *niaD* ORF.

pCM6 is a derivative of pEBO6 in which the *pyr4* gene substitutes for the *yA* gene; its construction is detailed below.

pEC62 and pNI160, carrying the *niaD A. nidulans* gene, disrupted by the *Fot1* and *impala* elements of *F. oxysporum*, respectively, have been described previously. pEC62 carries *Fot1* inserted in the third intron of the *niaD* gene (Migheli *et al.*, 1999); pNI160 carries *impala* inserted in the promoter region of the *niaD* gene (Hua-Van *et al.*, 2000).

pCM3 was constructed as follows: pEB06 was restricted to obtain a 3543 bp *Xho*I (−913 bp in relation to the ATG)–*Hpa*I (+2630 bp) containing a *yA* functional gene, including its promoter. This fragment was cloned in the unique *Nhe*I site of the *impala* 3′ non-coding region in the pNI160 plasmid (Fig. 6). This construction results in a 4.8 kb *impala*::*yA* autonomous transposable element.

pCM5 was constructed as follows: the 0.9 kb *Xho*I (+136 bp)–*Sty*I (+1027 bp) fragment of the *impala* transposable element ORF in pNI160 plasmid was replaced by the 3543 bp *Xho*I–*Hpa*I fragment of the *yA* gene (described above). This construction results in a 3.9 kb *impala*::*yA* defective transposable element (Fig. 6).

pImpE is a pMLC28-based plasmid containing the full-length autonomous *impE* copy in a 5 kb *Sph*I genomic insert (Hua-Van *et al.*, 2000).

Plasmid pEO6 was constructed using plasmid pNOM102 (Roberts *et al.*, 1989). This plasmid contained the *uidA* ORF as a *Nco*I fragment located between the *gpdA* promoter and the *trpC* terminator of *A. nidulans*. The *uidA* ORF was replaced by the ORF of ImpE, amplified by PCR using primers NORF for the N-terminal side and CORF for the C-terminus (primer sequences are given in Table 6). Both primers contained a *Nco*I site. After digestion with *Nco*I and alkaline phosphatase treatment of pNOM102, the vector carrying the *gpdA* promoter and the *trpC* terminator was ligated to the 1 kb *Nco*I-digested PCR product of *impE* ORF (DNA ligation system; Amersham). Orientation of the insert was checked by PCR, and individual clones were sequenced in order to confirm the absence of any PCR-induced mutations.

Table 6. Primer sequences.

Primers	Sequence 5′ → 3′
NORF[a]	5′-ctcaccatggctcgaggcaaggaacttacg-3′
CORF[a]	5′-cctaccatggtcaattagaacagccttc-3′
PL1	5′-gttttcgtctcatttcgtcagatcc-3′
TC19N	5′-ggtctctctatccggtgtcc-3′
TR1	5′-cgagttcgcgttagttcaagcc-3′
TR2	5′-ggtaatgccccgtgcctttgg-3′
9770	5′-gcgaagacctcagaccagacc-3′
OL145	5′-ctttcttcccggccaaagcctcg-3′
OL144	5′-gttcatgccgtggtcgctgc-3′
ON3	5′-ttgagggcatggccatca-3′
ON3R	5′-ctcgagttccttcggcaa-3′
ON8	5′-gagcttgtcaactcaaagctt-3′
16bis	5′-ccgtggtctggtctgaggtc-3′
GL	5′-ccagttcatgccgtggtcgct-3′
827	5′-cgttgaaaagccaatcaccctcag-3′
1630	5′-ggttattcttcgtccgcctc-3′
PF1	5′-gctctgtctactgcgctctt-3′
ON6R	5′-cgtgaggtctactcagtga-3′
U22	5′-acgccttcattacggtcacg-3′
L948	5′-tagtctcacaaaggttcttc-3′

a. Underlined, *Nco*I site.

Strains and growth conditions

Culture conditions for *A. nidulans* have been described by Pontecorvo *et al.* (1953) and Cove (1976). Culture conditions for *F. oxysporum* have been described by Deschamps *et al.* (1999).

Strains detailed below carry the following markers in different combinations (the following markers result in the requirements indicated): *argB2*, arginine; *biA1*, biotin; *pabaA1*, *para*-aminobenzoic acid; *pantoB100*, pantothenic acid; *pyrG89*, uracil or uridine; *riboB2* and *riboC3* riboflavin; *niaDΔ353*, deletion of the nitrate reductase gene, nitrate non-user (see below); *yA2*, yellow conidia; *yAΔ::pyr4*, yellow conidia, replacement of the *yA* gene with the *N. crassa pyr4* gene (see below); *camA112*, mitochondrially inherited chloramphenicol resistance (Gunatilleke *et al.*, 1975).

The following *A. nidulans* Glasgow laboratory strains were used. The CS2774 strain (*pantoB100*, *biA1*, *argB2*) was the recipient strain used to delete the *niaD* gene (see below). The transformant strain obtained, *pantoB100*, *biA1*, *argB*::pFB39 carrying the *niaD* deletion (*niaDΔ353*), was crossed with a *yA2*, *riboB2* strain in order to isolate a *yA2*, *riboB2*, *pantoB100*, *niaDΔ353*, named CS2708, used as a host strain to plasmids carrying the heterologous transposons (see below).

Transformants Trf1, Trf2 and Trf3 (see Table 1) carrying multiple copies of the *niaD* transgene interrupted by the *Fot1*

transposable element were obtained by co-transformation of the CS2708 strain with the pEC62 and pPL5 plasmids. They are phenotypically *riboB*$^+$.

The CS2767 strain (*riboB2*, *biA1*, *niaDΔ353*) carrying one copy of the *niaD* transgene interrupted by the *Fot1* transposable element was obtained by outcrossing Trf2 and CS2707.

The transformant Tri11, carrying three copies of the *niaD* transgene interrupted by the *impala* transposable element, was obtained by co-transformation of the CS2708 strain with the pNI160 and pPL5 plasmids (the transformant is phenotypically *riboB*$^+$).

The strain CS2780 (*biA1*, *pantoB100*, *niaDΔ353*), carrying one complete copy plus one additional copy with internal rearrangements of the *niaD* transgene interrupted by the *impala* transposable element, was obtained by crossing Tri11 and CS2707.

Transformants Tryi100 and Tryi101 were obtained by co-transformation of the CS2708 strain with the pCM3 (*impala::yA* element) and pPL5 plasmids. These last two strains were selected for further work as they carry single copies of pCM3. These strains are phenotypically *riboB*$^+$ and *yA*$^+$.

The recipient CS1268 strain (*pyrG89*, *pantoB100*, *pabaA1*) was used in order to delete the *yA* gene (see below).

The genotype of the resulting strain, named CS2751, is *yAΔ::pyr4*, *pyrG89*, *pantoB100*, *pabaA1*. CS2707 (*riboB2*, *biA1*, *argB2*, *niaDΔ353*) was crossed with the CS2751 strain in order to obtain CS2752 (*yAΔ::pyr4*, *niaDΔ353*, *riboB2*, *argB2*, *pabaA1*), CS2753 (*yAΔ::pyr4*, *niaDΔ353*, *riboB2*, *pabaA1*) and CS2755 (*yAΔ::pyr4*, *riboB2*, *pabaA1*).

CS2752 was used for co-transformation experiments with the pCM3/pFB39 and pCM5/pFB39 plasmids (transformants are phenotypically *yA*$^+$ and *argB*$^+$).

The CS2773 strain carrying a single copy of pCM3, deleted for the *yA* and *niaD* genes and *pabaA1*, was obtained by crossing Tryi100 with CS2753.

The CS2755 strain was co-transformed with the pCM3 8 kb *Sph*I–*Sph*I fragment in order to integrate the transposable element in the *niiA–niaD* resident bidirectional promoter (transformants are phenotypically *yA*$^+$ *and niaD*$^-$). The transformants were directly selected for resistance to 100 mM sodium chlorate (Cove, 1976). After purification, the positive transformants were checked for their inability to grow on nitrate and their ability to grow on nitrite and hypoxanthine as sole nitrogen sources. This shows that the resistance to chlorate does not result from independent *nirA* or *cnx* spontaneous mutations (Cove, 1976). The presence of a single copy of the transposon in the resident promoter of the *niaD* gene was ascertained by Southern blots after restriction with appropriate enzymes. One of these strains selected for further work was called CS2778.

CS2752, co-transformed with pImpE/pFB39 or pE06/pFB39, gave CS2776 and CS2777, respectively, carrying a single copy of the *trans*-activator element. The presence of single copies of the transposon-carrying plasmid was ascertained in each case by Southern blots after restriction with appropriate enzymes.

Strains CS2900 (*biA1*, *riboC3*, *niaDΔ353*, *camA112*) (Gunatilleke *et al.*, 1975), CS2708, CS2779 and Tryi100 were used for the hybrid dysgenesis experiments.

Wild-type strain isolates different from the standard Glasgow strain are Birmingham strains G001, G0068 and AJC10.16.

Two *F. oxysporum* strains were used: *niaD-37* and *Fot15* (Deschamps *et al.*, 1999).

Deletion of the niaD *gene*

The CS2773 strain (*pantoB100*, *biA1*, *argB2*) strain was co-transformed with a linear fragment derived from bAN353 (see below) and pFB39 carrying the *argB*$^+$ allele. The 1.8 kb *BamH*I–*Hin*dIII linear fragment carries a 0.3 kb *niaD* promoter sequence ligated to the 3′ *niaD* flanking region as described above. This generates a sequence deleted from 55 nucleotides upstream of the initiation codon of the *niaD* genes to 2728 bp downstream. A total of 2768 bp are thus deleted, including the *impala* insertion site. The transformants *pantoB100*, *biA1*, *argB::*pFB39, *niaDΔ353* were selected on chlorate medium (see above) lacking arginine. These strains were checked for their inability to use nitrate as a nitrogen source. One strain showed the restriction pattern expected by the deletion of the 2728 nucleotides. This was confirmed by sequencing. The resulting deletion was called *niaDΔ353*.

Deletion of the yA *gene*

pEB06 was restricted with *Xho*I and *Hpa*I in order to eliminate a 3543 bp *Xho*I (−913 bp in relation to the ATG)–*Hpa*I (+2630 bp) from *yA*. Deleted sequences include the ORF and promoter regions (Aramayo and Timberlake, 1990). After religation, the pCM2 plasmid was obtained. A 3.2 kb *Bgl*II–*Bgl*II fragment encompassing the *pyr4* gene of the pFB6 plasmid was cloned in pCM2 digested by *Sma*I. This site is upstream of the deleted sequences. The resulting plasmid is called pCM6. This plasmid carries a 5.7 kb *Nru*I–*Nru*I DNA fragment, which contains the 1.2 kb fragment of the *yA* gene upstream region followed by the 3.2 kb *pyr4* gene fragment, followed by an additional 300 bp of upstream region and then 1 kb of the *yA* downstream region. 'Upstream' and 'downstream' are defined here in relation to the deleted regions.

The *yA* gene deletion was carried out in the CS1268 strain (*pyrG89*, *pantoB100*, *pabaA1*) by transformation with the linear 5.7 kb *Nru*I–*Nru*I DNA fragment obtained from the pCM6 plasmid. Thus, a *yAΔ::pyr4* substitution event was obtained. In this event, 3543 bp spanning from 913 bp upstream of the ATG of *yA* to 2630 nucleotides downstream are deleted. The resulting strain was called CS2751. The presence of the substitution event was confirmed by Southern blots, PCR and by the co-segregation of the *yA*$^-$ and *pyr4*$^+$ phenotypes in crosses.

Phenotypic excision test

In order to estimate the frequency of transposon excision, 10^4–10^8 conidia were spread on minimal medium with 10 mM sodium nitrate as nitrogen source. The cultures were incubated at 37°C for 5 days. In parallel, serial dilutions were plated on minimal medium containing 5 mM ammonium

D(+) tartrate as sole nitrogen source to estimate the total number of viable conidiospores.

Co-transformation procedures

Protoplast preparation and transformation procedures were performed according to the method of Tilburn *et al.* (1983). pEC62 (5 μg) or pNI160 (5 μg) plus 1 μg of pFB39 were used for each co-transformation of the CS2708 strain. Samples of 5 μg of pCM3, pCM5, pImpE or pE06 plus 1 μg of pFB39 or pPL5 were used for each co-transformation of CS2708 and CS2752 strains.

Nucleic acid manipulations

Cloning and subcloning. All cloning procedures, including plasmid preparation from *Escherichia coli*, ligation and transformation were performed according to the methods of Sambrook *et al.* (1989).

DNA extraction. DNA from *A. nidulans* was extracted as described by Specht *et al.* (1982). Plasmid DNA from *E. coli* was prepared as described by Sambrook *et al.* (1989).

Southern blot analysis. For Southern hybridization, 5 μg of DNA was digested with a suitable endonuclease, fractionated through a 0.7% agarose gel and transferred by standard methods (Sambrook *et al.*, 1989) onto Hybond-N nylon membranes by UV cross-linking. DNA probes were either PCR products amplified by specific primers or enzyme restriction fragments obtained from plasmids. The P2608 probe was a PCR product obtained using the specific primers TR1 and TR2 internal to transposon sequences. The *niaD* probe was a PCR product amplified by specific primers 9770 and OL145 (Table 6). The *Fot1*-specific probe was a 0.9 kb *Bam*HI transposon internal fragment obtained by pEC62 enzymatic digestion. The *impala*-specific probe was a 1.09 kb *Pst*I transposon internal fragment obtained from pNI160. These probes were ^{32}P labelled with a random primer extension kit (Amersham Life Science). Hybridization was carried out according to the manufacturer's conditions and in high-stringency conditions (65°C). When multiple transforming sequences were detected in Southern blots by restriction patterns, their number was estimated using a Storm 80 Phosphorimager using a single-copy strain as a control.

RNA extraction and Northern blot analysis. Total RNA was isolated as described by Lockington *et al.* (1985), denatured by glyoxal treatment and separated by electrophoresis on 1% agarose gel as described by Sambrook *et al.* (1989). The *Fot1*-specific probe (described before) was labelled using a random primer extension kit (Amersham Life Science). The probe corresponding to the *A. nidulans Fot1*-like element was obtained by PCR amplification of an internal sequence of the *A. nidulans Fot1*-like transposon. To this end, the primers TR1 (situated 56 bp downstream from the 5′ ITR) and TR2 (situated 117 bp upstream from the 3′ ITR) were used (Table 6). An *acnA* probe (Fidel *et al.*, 1988) was used as a control for RNA loading. In Northern blots in which RNAs from both organisms were compared, the loading was assessed by colouring the blot with methylene blue according to the method of Wilkson *et al.* (1990).

PCR amplification. Amplifications were carried out in a Perkin-Elmer Cetus thermal cycler. Total genomic DNA (300 ng) was denatured for 1 min 30 s at 95°C, annealed to specific primers for 1 min at an appropriate temperature and extended at 72°C. For each reaction, 0.5 units of *Taq* polymerase (Promega) was used. The extension time varied depending on the size of the fragments to be amplified. The primers used are shown in Table 6.

NORF and CORF were used to amplify the ORF of the *impE* transposable element.

Primers PL1 and TC19N, deduced from terminal regions of the *niaD* gene, were used for PCR amplification and for sequencing the *niaD* deletion.

A specific *niaD* probe was obtained by PCR on total genomic DNA. Primers used for *niaD* amplification and deduced from the gene sequence were 9770 and OL145.

PCR reactions were carried out on *niaD* and *nirA* genes carrying spontaneous mutations in order to detect possible transposon insertions in these genes. To this end, specific *niaD* and *nirA* primers were used. The *nirA* gene was amplified by two pairs of primers, ON3/ON3R and ON8/ON6R. The whole gene can be monitored using these primers, from 313 nucleotides upstream of the start translation signal up to 3255 nucleotides downstream, at the end of the ORF. The *niaD* gene was amplified by primers OL144 and OL145 (which amplify a *niaD* region starting in the *niaD* promoter region, 54 bp upstream from the ATG translation codon, and finishing 2759 nucleotides downstream from the ATG).

U22 and L948 were used to amplify the ORF of the *imp160* transposable element.

The primer sequences are given in Table 6.

Selection of niaD *and* nirA *mutants*

Spontaneous *niaD*$^-$ and *nirA*$^-$ mutants were isolated by selection for chlorate resistance on minimal medium and assigned to either gene as described by Cove (1976).

DNA sequencing

In order to sequence the *niaD* deletion (*niaDΔ353*), genomic DNA from the transformant strain carrying the deletion was amplified by PCR with specific primers (PL1 and TC19N; Table 6) identical to regions of the *niaD* gene still present in the *niaDΔ353* deletion. The amplification product obtained, including the *niaD* internal deletion (0.28 kb versus 3.1 kb in the *niaD* wild-type strain), was sequenced using the Sequenase PCR product sequencing kit (Amersham) according to the manufacturer's instructions.

Footprints left by transposon excision were analysed by sequencing the PCR product obtained using primers identical to *niaD* regions surrounding the transposon insertion site. To sequence footprints left by excision of *impala*, primers were PL1, 16bis and GL (Table 6). For *Fot1*, primers were 827, 1630 and PF1 (Table 6). Primers GL and PF1, placed a few basepairs from the *impala* and *Fot1* excision sites, respectively, were also used in the sequencing reaction.

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